Effect of different microalgal n-3 PUFA supplementation doses on yolk color and n-3 LC-PUFA enrichment in the egg.

Charlotte Lemahieu[a,1,*], Charlotte Bruneel[a,1], Romina Termote-Verhalle[a,1], Koenraad Muylaert[b], Johan Buyse[c,1], and Imogen Foubert[a,1]

[a]Research unit Food and Lipids, KU Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium.

* Corresponding author: Charlotte Lemahieu

Phone: +32 (0) 56 24 64 14

Fax: +32 (0) 56 24 69 99

E-mail: charlotte.lemahieu@kuleuven-kulak.be

[b]Research unit Aquatic Biology, KU Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium.

[c]Division of Livestock-Nutrition-Quality, KU Leuven, Kasteelpark Arenberg 30, 3001 Leuven, Belgium.

[1]Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven, Kasteelpark Arenberg 20, 3001 Leuven, Belgium

ABSTRACT

To examine the effect of different omega-3 polyunsaturated fatty acids (n-3 PUFA) doses on the long chain (LC) n-3 PUFA enrichment in the egg yolk, *Isochrysis galbana* was supplemented in nine different doses (from 0 – 400 mg n-3 PUFA per 100 g feed) to the diet of laying hens. A linear increase of the n-3 LC-PUFA enrichment was observed up to a dose of 120 mg n-3 PUFA per 100 g feed. Higher n-3 PUFA supplementation doses resulted in a decreased efficiency of n-3 LC-PUFA incorporation into the egg yolk. Pronounced color changes were observed due to transfer of carotenoids from the microalgal biomass to the egg yolk. However, a corresponding linear trend in yolk color changes was not observed for the low supplementation doses when compared to the n-3 LC-PUFA changes in the egg yolk. The *a**-value increased remarkably with supplementation of 3% *Isochrysis* (150 mg n-3 PUFA per 100 g feed) and remained stable with the higher supplementation doses.

KEYWORDS

Omega-3 polyunsaturated fatty acids

Microalgae

Isochrysis galbana

n-3 PUFA supplementation dose

Enrichment

Egg

HIGHLIGHTS

- Linear increase of n-3 LC-PUFA content in the egg up to 120 mg n-3 PUFA/100 g feed
- Decreased efficiency of n-3 LC-PUFA incorporation above 120 mg n-3 PUFA/100 g feed
- No linear trend in yolk color changes was observed with low level microalgal dosing
- Redness of yolk increased remarkably with supplementation of 3% *Isochrysis*

ABBREVIATIONS

n-3 LC-PUFA	Omega-3 long chain polyunsaturated fatty acids
ALA	α-Linolenic acid
SDA	Stearidonic acid
EPA	Eicosapentaenoic acid
DPA	Docosapentaenoic acid
DHA	Docosahexaenoic acid

1. Introduction

Enrichment of eggs with omega-3 polyunsaturated fatty acids (n-3 PUFA) is possible through supplementation of the feed of laying hens with n-3 PUFA sources (e.g., flaxseed, fish oil or even microalgae) [1]. However, a clear distinction must be made between the enrichment of eggs with the shorter chain ($C \leq 18$) PUFA (α-linolenic acid or ALA) and n-3 longer chain PUFA or n-3 LC-PUFA (eicosapentaenoic acid or EPA and docosahexaenoic acid or DHA) [1]. Since several health benefits are related to the content of n-3 LC-PUFA instead of ALA, it is more interesting to raise the level of n-3 LC-PUFA in the egg [2-5].

ALA enriched eggs can be obtained by the supplementation of the diet of laying hens with flaxseed[1,6]. Enrichment of eggs with n-3 LC-PUFA can be achieved by supplementing the feed with fish oil, a source of EPA and DHA. A more recent trend is to replace fish oil with heterotrophic and autotrophic microalgae [1,4,5]. Supplementation of diets with autotrophic microalgae provides the most environmentally sustainable approach to raising n-3 PUFA concentrations in eggs. Unlike heterotrophic microalgae, autotrophic microalgae only need light and CO_2 to form their biomass and the desirable fatty acids.

To the best of our knowledge, the effect of different microalgal n-3 PUFA supplementation doses on the n-3 LC-PUFA enrichment in the egg yolk has rarely been examined. Herber and Van Elswyk [7] supplemented 2.4 % and 4.8 % heterotrophically cultivated microalgae to the diet of laying hens, which corresponds with a supplementation of 200 mg DHA/day and 400 mg DHA/day respectively. They noted that doubling the amount of microalgae in the diet did not result in a doubled n-3 LC-PUFA deposition in the egg yolk, thus leading to a decreased efficiency of n-3 LC-PUFA incorporation with higher n-3 PUFA supplementation doses. The same observation was made by Bruneel et al. [8] who supplemented two doses of the autotrophic microalgae *Nannochloropsis*, 5 % and 10 %, corresponding with an intake of 76

mg n-3 PUFA per 100 g feed and 152 mg n-3 PUFA per 100 g feed. They obtained a n-3 LC-PUFA enrichment efficiency of 25 % and 20 % for the respective doses of 5% and 10% *Nannochloropsis*. A lower n-3 LC-PUFA incorporation efficiency was also observed in our previous study [4], where four different microalgae in two different doses (125 mg and 250 mg ALA+EPA+DHA per 100 g feed) were supplemented to the hens' diet. Carvalho et al. [9] supplemented different amounts of DHA to the diet of the laying hens by the addition of salmon oil or the heterotrophic microalga *Schizochytrium*. These researchers concluded that the highest DHA incorporation efficiency was obtained at an intake of 120 mg to 180 mg DHA per 100 g feed. Remarkable decreases in incorporation efficiencies were observed with higher supplementation doses. However, in this study, no information was provided for supplementation doses lower than 20 mg DHA per 100 g diet. Nitsan et al. [10] supplemented very low doses of *Nannochloropsis*, corresponding to very low doses of n-3 PUFA. Addition of 0.1 % and 0.5 % *Nannochloropsis* did not give rise to enrichment of DHA in the egg yolk [10]. Only 1% supplementation of *Nannochloropsis* gave rise to a higher n-3 PUFA content in the egg yolk. From the above, it is clear that supplementation of different microalgal doses, corresponding with different n-3 PUFA doses, has a significant effect on the n-3 LC-PUFA enrichment in the egg yolk.

An important difference between the use of fish oil and microalgae for supplementation of laying hens' feed with n-3 LC-PUFA is that microalgae contain significant concentrations of carotenoids. Like the n-3 LC-PUFA, the carotenoids can be transferred to the egg yolk and influence the yolk color [4]. This can be advantageous, because it reduces the need for addition of synthetic carotenoids to the feed, which is a significant cost. However, it is important to take into account consumer preferences relating to the yolk color, as deeply red yolk colors are not desirable [5].

Unfortunately, in the above mentioned studies, only a few different n-3 PUFA supplementation doses were studied, making it difficult to draw conclusions on the general effect of doses on the n-3 PUFA and/or n-3 LC-PUFA enrichment(efficiency) in the egg yolk. Moreover, in most cases, no information was provided on the n-3 PUFA dose, making it difficult to compare different studies. Therefore, in this research, for the first time, a thorough study was performed to examine the effect of nine n-3 PUFA supplementation doses on the n-3 LC-PUFA incorporation(efficiency) in the egg yolk using supplementation of different doses of *Isochrysis galbana* into the diet of the laying hens. In addition to the n-3 LC-PUFA enrichment, the effect of the different *Isochrysis* doses on the egg yolk color was examined in this study, since earlier research has shown a carotenoid transfer from autotrophic microalgal biomass to the egg yolk [4].

2. Materials and methods

2.1. Microalgal biomass

To determine the impact of different supplementation doses on the n-3 LC-PUFA enrichment in the egg yolk, *Isochrysis galbana*, selected by Lemahieu et al. [4] as the most appropriate autotrophic microalgal species, was supplemented into the diet of laying hens. The *Isochrysis galbana* lyophilized biomass used in this study was obtained from Archimede Ricerche (Italy).

The total lipid content and the fatty acid profile of the *Isochrysis* biomass were determined in triplicate by the method described in Ryckebosch et al. [11]. After the extraction and quantification of the total lipids, the fatty acids were methylated and the fatty acid methyl esters were gas chromatographically separated (Trace GC Ultra, Thermo Scientific, Interscience, Louvain-la-Neuve, Belgium), as described in Lemahieu et al. [4].

2.2. Animals and diets

72 ISA Brown laying hens (33 weeks of age, ‘t Munckenei, Wingene, Belgium) were housed in battery cages (two hens per cage) under environmentally controlled conditions (room temperature of 20 °C and a 16 h photoperiod). Feed and water were supplied for *ad libitum* consumption. The experiment started with an adaptation period of 14 days in which the hens could adapt to the new environmental conditions and the new commercially available control diet (AVEVE legmeel Total 277). The control diet consisted mainly of corn, wheat, sunflower cake, corn gluten, limestone, soybean meal, and palm oil. The control diet contained no n-3 LC-PUFA, and only a small amount of ALA (0.074%) was observed (**Table 2**).

After the adaptation period, the laying hens were randomly assigned to one of the nine treatment diets (n = 8 hens per treatment diet): a control diet, without supplementation of *Isochrysis galbana*, and eight diets in which the amount of *Isochrysis* was calculated to reach eight

138 different levels of extra microalgal n-3 PUFA (ALA + stearidonic acid (SDA) + EPA +
139 docosapentaenoic acid (DPA) + DHA) supplementation per 100 g feed. An overview of the
140 different n-3 PUFA doses and the added levels of *Isochrysis galbana* is shown in **Table 1**. The
141 supplementation of *Isochrysis* in different doses lasted 21 days. Earlier research by Lemahieu
142 et al. [12] showed a maximum n-3 PUFA incorporation after 14 days of supplementation.
143 However, to be sure of the maximum incorporation of the n-3 LC-PUFA in this experiment,
144 *Isochrysis* was supplemented for 21 days to obtain reliable results.

145 During the supplementation period, some zootechnical parameters and egg quality parameters
146 were registered on a daily basis: feed intake, egg production, mortality, morbidity, egg weight
147 and yolk weight.

148 ### 2.3. Egg collection, storage and analysis

149 The eggs were collected on a daily basis and stored at -20 °C. The eggs at the start and the end
150 of the supplementation period were analyzed to determine the n-3 LC-PUFA content and the
151 yolk color according to the methods described in Lemahieu et al. [4].

152 ### 2.4. Statistical analysis

153 The results were statistically evaluated by one way analysis of variance (ANOVA) and post-
154 hoc Tukey's test with α=0.05.

155

3. Results and discussion

3.1. Microalgal biomass

The fatty acid profile of *Isochrysis galbana* was determined in order to calculate the different supplementation doses (**Table 1**). The n-3 PUFA composition of *Isochrysis* is shown in **Table 2**. Predominantly DHA was observed as n-3 LC-PUFA in the biomass but *Isochrysis* also contained high amounts of SDA. So, instead of only taking into account ALA, EPA and DHA as omega-3 fatty acids, as in the experimental design of Lemahieu et al. [4], all the omega-3 fatty acids (ALA, SDA, EPA, DPA and DHA) were taken into account to calculate the supplemented dose of *Isochrysis galbana* (**Table 1**). The added levels of *Isochrysis galbana* varied between 0 % and 8.1 % to reach n-3 PUFA supplementation doses of 0 to 400 mg per 100 g feed.

3.2. n-3 LC-PUFA enrichment in the egg yolk

Supplementation of *Isochrysis* to the diet of the laying hens resulted in changes of the n-3 PUFA content (**Table 1**), no drastic changes in other fatty acids were observed (results not shown). **Figure 1** shows the n-3 LC-PUFA content (EPA+DPA+DHA) in the egg yolk at the start of the supplementation period (day 0) and at the end of the supplementation period (day 21) for the different doses of microalgal n-3 PUFA supplementation. These results show, at day 21, a linear relationship between the two parameters until a dose of 120 – 150 mg n-3 PUFA per 100 g feed. This linear increase of the n-3 LC-PUFA enrichment in the yolk was mainly caused by DHA enrichment, as DHA is preferentially incorporated in the egg yolk [4]. More than 90% of the n-3 LC-PUFA content in the egg yolk was attributed to the fatty acid DHA. A flattening of the curve was observed at higher n-3 PUFA supplementation doses, indicating in a decrease in efficiency of the n-3 LC-PUFA incorporation. In **Table 3**, the efficiency of n-3 LC-PUFA

incorporation in the egg for several microalgal supplementation doses is shown. To obtain this value, the ratio of the n-3 LC-PUFA enrichment in the egg and the intake of n-3 PUFA was calculated. The former value was obtained by correcting the n-3 LC-PUFA content in the egg at day 21 with the n-3 LC-PUFA content in the egg at day 0. The latter value was calculated by multiplying the different doses (for example 30 mg n-3 PUFA per 100 g feed) by the mean feed intake of the laying hens in the respective groups [4]. The highest efficiency of n-3 LC-PUFA incorporation was observed with supplementation of 120 mg microalgal n-3 PUFA per 100 g feed, although this value was not significantly different from the efficiencies obtained by supplementation of 60 mg and 90 mg microalgal n-3 PUFA per 100 g feed. However, the total n-3 LC-PUFA enrichment is equally important. As supplementation of 120 mg n-3 PUFA per 100 g feed (corresponding with the supplementation of 2.4% *Isochrysis* biomass) leads to the highest n-3 LC-PUFA enrichment in the yolk with the highest n-3 LC-PUFA incorporation efficiency, this supplementation dose can be considered as the optimal dose (**Table 3**).

A linear increase of n-3 PUFA content in the egg yolk by supplementation of different n-3 PUFA doses was also observed by Caston & Leeson [13], Schiedeler & Froning [14] and Van Elswyk [6], when flaxseed was used as the supplement. However, in these studies, no information was given about the exact amount of n-3 PUFA supplementation. So, it is hard to say if the linear increment corresponds to similar observations in this study. On the other hand, many studies, supplementing different amounts of n-3 PUFA to the diet of laying hens, observed a decrease in efficiency of n-3 PUFA incorporation with increasing n-3 PUFA supplementation. Van Elswyk [6], for example, supplemented different doses of fish oil as a source of n-3 PUFA, and observed no significant difference in n-3 PUFA content of the eggs from hens fed 15 g fish oil/kg or 30 g fish oil/kg. This means that starting from a certain dose, the n-3 PUFA content in the egg reached the maximum incorporation despite the increase of n-

3 PUFA in the diet of laying hens. However, no information was given about the fish oil composition, so the effective supplemented n-3 PUFA dose could not be determined. The same doses of fish oil (15 g/kg diet and 30 g/kg diet, corresponding to a supplementation of 462 mg n-3 PUFA and 924 mg n-3 PUFA per 100 g feed, respectively) were used by Cachaldora et al. [15]. The supplementation of these two doses of fish oil resulted in an efficiency of n-3 LC-PUFA incorporation of approximately 22 % and 18 % for the respective doses, which means that a higher dose also resulted in a lower efficiency of n-3 LC-PUFA enrichment. However, only two doses were tested so the general trend could not be derived.

The same trend was observed by supplementation of autotrophic microalgae as the n-3 PUFA source. Bruneel et al. [8] also observed a decreased efficiency of n-3 LC-PUFA incorporation for the highest n-3 PUFA supplementation dose (152 mg n-3 PUFA per 100 g feed) in comparison with the lower dose (76 mg n-3 PUFA per 100 g feed). This corresponds also with the study of Lemahieu et al. [4], who suggested that the highest incorporation efficiency can be obtained with a supplementation dose below 250 mg n-3 PUFA per 100 g feed.

3.3. Yolk color analysis

Not only was an enrichment of n-3 LC-PUFA observed with supplementation of autotrophic microalgae into the diet of the laying hens, but also a remarkable yolk color change [4]. The egg yolk color is influenced by the type and concentration of carotenoids present in the diet of the laying hen [16-18]. Therefore, supplementation of autotrophic microalgae, rich in carotenoids, results in changes of the yolk color [4]. The results of the yolk color analysis are shown in **Table 4**. The yolk color was measured with a colorimeter to obtain a CIE 1976 *L**, *a**, *b** color space. The *L**-value corresponds to the degree of lightness, the *a**- value corresponds to the degree of redness, and the *b**- value, corresponds to the yellowness of the egg yolk [8]. In general, addition of *Isochrysis* to the diet of laying hens resulted in a decrease

of the *L** - and *b** -values and an increase of the *a**-value. This corresponds with an increased darkness, reduced yellowness and increased redness of the egg yolk. Based on the research of Lemahieu et al. [4], the color shift by supplementation with *Isochrysis* can be explained by the enrichment of fucoxanthin derivatives in the egg yolk. Fucoxanthin has a typical brown color, which is darker than the yellow lutein and zeaxanthin pigments normally present in the egg yolk.

When looking in more detail at the effect of dose on the redness of the egg yolk, it can be seen that the *a**-value remained more or less the same up to a supplementation of 120 mg microalgal n-3 PUFA per 100 g feed (2.4% *Isochrysis* supplementation). Starting from 150 mg n-3 PUFA per 100 g feed, corresponding with 3% *Isochrysis* supplementation, a remarkable increase of the *a**-value was observed. Higher supplementation doses did not further increase the *a**-value. However, significant changes in *L**-, *a**- and *b**-values may be perceived differently by the human eye, we therefore scored the yolk color using Roche values as well. A significant increase of the Roche value was observed starting from a supplementation of 150 mg microalgal n-3 PUFA per 100 g feed corresponding with 3% *Isochrysis* biomass supplementation. Doses of ≥ 200 mg n-3 PUFA per 100 g feed even reached a Roche value of ≥15. In Belgium, egg yolks with a definite orange tinge (corresponding to a Roche value of 12-13) are desired. Higher Roche values (corresponding to deeply red egg yolk) lead to a reduced consumer acceptability [5]. In the present study, supplementation with up to 3% *Isochrysis* resulted in acceptable yolk color.

Leeson & Caston [16] examined the effect of different doses of lutein supplementation to the diet of the laying hens. They observed a dramatic increase of the yolk color, which was, however, little affected by the supplementation dose. The yolk color leveled off at *a**-values between 13 and 14 and was unaffected by dietary lutein supplements above 250 ppm. This trend

corresponds to the results obtained in this study: no further increase of the *a**-value was observed when adding more than 3% *Isochrysis* biomass (*a**-value of approximately 19). However, this is in contrast with the yolk color results obtained by Bruneel et al. [8] and Fredriksson et al. [19], who supplemented *Nannochloropsis* in two different doses. The *a**-values increased significantly with increased supplementation of *Nannochloropsis*. This observation was also made by Grashorn and Steinberg [20] who supplemented different levels of canthaxanthin to the diet of laying hens and observed increased *a**-values with increasing canthaxanthin concentration. The different observations made by different researchers can presumably be explained by the different amount and types of carotenoids supplemented to the diet, for example lutein versus canthaxanthin. In addition, the different matrix in which they are supplied can also have an influence, for example the supplementation of the pure carotenoids or addition of, for example, microalgal biomass, where the bioaccessibility is an important parameter to take into account.

3.4. Zootechnical performance of the laying hens and egg quality parameters

No drastic impact on the zootechnical performance parameters (feed intake, egg production, mortality and morbidity) was observed by supplementation of different doses of n-3 PUFA to the diet of laying hens (**Table 5**). Only a significantly lower feed intake was observed with the supplementation of 60 mg microalgal n-3 PUFA per 100 g feed. However, this can be explained by the fact that one hen got sick in this experimental group. Also no drastic effects were observed on the egg and yolk weight (**Table 5**). It can thus be concluded that different supplementation doses have no impact on the zootechnical performance of the laying hens and the egg quality parameters, which is in accordance with Bruneel et al. [8] and Lemahieu et al. [4].

4. Conclusion

Supplementation of different n-3 PUFA doses of *Isochrysis* to the diet of laying hens linearly increased the n-3 LC-PUFA content in the egg yolk up to a supplementation dose of 120 mg microalgal n-3 PUFA per 100 g feed. Higher supplementation doses resulted in a flattening of the amount of n-3 LC-PUFA incorporation, reflecting a decreased efficiency of n-3 LC-PUFA enrichment in the egg yolk. Due to the carotenoid rich biomass that was supplemented, drastic yolk color changes were observed, albeit there was no linear increase of the *a** value observed. Between 30 and 120 mg n-3 PUFA per 100 g feed, more or less the same *a**-value was observed, while from 150 mg n-3 PUFA per 100 g feed (3% microalgal biomass) a jump of this value was observed

Based on the n-3 LC-PUFA enrichment in the egg yolk and the n-3 LC-PUFA incorporation efficiency, 120 mg n-3 PUFA per 100 g feed can be considered as the optimal supplementation dose since higher n-3 PUFA supplementation doses (up to 400 mg n-3 PUFA per 100 g feed) lead to decreased n-3 LC-PUFA incorporation efficiencies in the egg yolk. To obtain this dose, 2.4 % of *Isochrysis* biomass has to be supplemented, which maintains an acceptable yolk color.

ACKNOWLEDGEMENTS

This publication has been produced with the financial support of Flanders' Food – IWT (OMEGA-EI). We wish to thank everybody who has contributed to this project, especially the companies of the Flanders' Food OMEGA-EI project, ILVO (for making the different experimental diets), Stefaan Verhelle ('t Munckenei, for delivery of the laying hens), Marcel Samain and André Respen (for taking care of the laying hens). Also thanks to Sam Eyley for reading the paper and correcting the grammatical English mistakes.

REFERENCES

[1] I. Fraeye, C. Bruneel, C. Lemahieu, J. Buyse, K. Muylaert, I. Foubert, Dietary enrichment of eggs with omega-3 fatty acids: A review, Food Res. Int. 48 (2012) 961-969.

[2] E.A. Trautwein, n-3 Fatty acids – physiological and technical aspects for their use, Sciences-New York. 103 (2001) 45-55.

[3] U. Gogus, C. Smith, n-3 Omega fatty acids: a review of current knowledge, Int. J. Food Sci. Technol. 45 (2010) 417-436.

[4] C. Lemahieu, C. Bruneel, R. Termote-Verhalle, K. Muylaert, J. Buyse, I. Foubert, Impact of feed supplementation with different omega-3 rich microalgae species on enrichment of eggs of laying hens, Food Chem. 141 (2013) 4051-4059.

[5] C. Lemahieu, C. Bruneel, R. Termote-Verhalle, K. Muylaert, J. Buyse, I. Foubert, Omega-3 long-chain polyunsaturated fatty acid enriched eggs by microalgal supplementation, Lipid Technol. 25 (2013) 204-206.

[6] M.E. Van Elswyk, Comparison of n-3 fatty acid sources in laying hen rations for improvement of whole egg nutritional quality: a review, Brit. J. Nutr. 78 (1997) S61-S69.

[7] S.M. Herber, M.E. Van Elswyk, Dietary marine algae promotes efficient deposition of n-3 fatty acids for the production of enriched shell egss, Poult. Sci. 75 (1996) 1501-1507.

[8] C. Bruneel, C. Lemahieu, I. Fraeye, E. Ryckebosch, K. Muylaert, J. Buyse, I. Foubert, Impact of microalgal feed supplementation on omega-3 fatty acid enrichment of hen eggs, J. Funct. Foods. 5 (2013) 897-904.

[9] A.P. Carvalho, C.M. Monteiro, F.X. Malcata, Simultaneous effect of irradiance and temperature on biochemical composition of the microalga Pavlova lutheri, J. Appl. Phycol. 21 (2009) 543-552.

[10] Z. Nitsan, S. Mokady, A. Sukenik, Enrichment of poultry products with omega3 fatty acids by dietary supplementation with the alga Nannochloropsis and mantur oil, J. Agric. Food Chem. 47 (1999) 5127-5132.

[11] E. Ryckebosch, K. Muylaert, I. Foubert, Optimization of an analytical procedure for extraction of lipids from microalgae, J. Am. Oil Chem. Soc. 89 (2012) 189-198.

[12] C. Lemahieu, C. Bruneel, R. Termote-Verhalle, K. Muylaert, J. Buyse, I. Foubert, Dynamics of n-3 LC-PUFA incorporation in the egg yolk by microalgal supplementation, In Preparation for Algal Research.

[13] L. Caston, S. Leeson, Research note: Dietary flax and egg composition, Poult. Sci. 69 (1990) 1617-1620.

[14] S.E. Schiedeler, G.W. Froning, The combined influence of dietary flaxseed variety, level, form and storage conditions on egg production and composition among vitamin E-supplemented hens, Poult. Sci. 75 (1996) 1221-1226.

[15] P. Cachaldora, P. García-Rebollar, C. Alvarez, J.C. De Blas, J. Méndez, Effect of type and level of basal fat and level of fish oil supplementation on yolk fat composition and n-3 fatty acids deposition efficiency in laying hens, Anim. Feed Sci. Technol. 141 (2008) 104-114.

[16] S. Leeson, L. Caston, Enrichment of eggs with lutein, Poult. Sci. 83 (2004) 1709-1712.

[17] F. Karadas, E. Grammenidis, P.F. Surai, T. Acamovic, N.H.C. Sparks, Effects of carotenoids from lucerne, marigold and tomato on egg yolk pigmentation and carotenoid composition, Br. Poult. Sci. 47 (2006) 561-566.

[18] D.E., Breithaupt, Xanthophylls in poultry feeding, in: G. Britton, Liaaen-Jensen, S., Pfander, H., Carotenoids – Volume 4: Natural Functions, Birkhäuser Verlag, Switserland, 2008, pp. 255-264.

[19] S. Fredriksson, K. Elwinger, J. Pickova, J., Fatty acid and carotenoid composition of egg yolk as an effect of microalgae addition to feed formula for laying hens, Food Chem. 99 (2006) 530-537.

[20] M.A. Grashorn, W. Steinberg, Deposition rates of canthaxanthin in egg yolks, Arch. Geflügelk. 66 (2002) 258-262.

Table 1: Overview of the nine different microalgal supplemented n-3 PUFA (ALA+SDA+EPA+DPA+DHA) doses (in mg/100 g feed) and the corresponding percentage of supplemented microalgal biomass (in %)

	Dose of microalgal ALA+SDA+EPA+DPA+DHA (mg/100 g feed)	**Supplemented microalgal biomass (%)**
Diet 1	0	0
Diet 2	30	0.6
Diet 3	60	1.2
Diet 4	90	1.8
Diet 5	120	2.4
Diet 6	150	3
Diet 7	200	4.1
Diet 8	300	6.1
Diet 9	400	8.1

353 **Table 2**: n-3 PUFA composition (ALA, SDA, EPA, DPA and DHA) of the control diet,
354 AVEVE legmeel Total 277, and *Isochrysis galbana* (in g/100 g biomass; mean ± SD; n=3).

n-3 PUFA	**Control diet**	***Isochrysis galbana***
ALA	0.074 ± 0.023	1.247 ± 0.016
SDA	0	1.830 ± 0.031
EPA	0	0.091 ± 0.004
DPA	0	0.023 ± 0.002
DHA	0	1.737 ± 0.052

355

Table 3: Incorporation efficiency[y] of the n-3 LC-PUFA (in %, mean ± SD; n = 8) in the egg yolk for the different supplementation doses of *Isochrysis galbana*.

Microalgal n-3 PUFA (mg/100g feed)	Intake of n-3 PUFA (mg)	Enrichment of n-3 LC-PUFA (mg)	Incorporation efficiency (%)
30	35.7 ± 0.1	14.7 ± 4.8[a]	41.1 ± 13.4[b]
60	68.6 ± 1.2	32.9 ± 6.0[b]	47.9 ± 8.8[bc]
90	107.0 ± 0.4	48.5 ± 6.6[c]	45.3 ± 6.2[bc]
120	142.0 ± 0.6	74.9 ± 6.0[d]	52.8 ± 4.2[c]
150	178.3 ± 0.7	73.3 ± 6.4[d]	41.1 ± 3.6[b]
200	237.6 ± 0.5	90.4 ± 5.6[e]	38.1 ± 2.4[b]
300	355.1 ± 2.4	101.1 ± 4.9[f]	28.5 ± 1.4[ab]
400	465.6 ± 2.8	129.0 ± 7.7[g]	27.7 ± 1.7[a]

[x] Results with the same letter in the same column are not significantly different ($p < 0.05$)

[y] The incorporation efficiency was calculated by taking the ratio of the n-3 LC-PUFA enrichment (in mg/egg; mean ± SD; n = 8) to the n-3 PUFA intake (in mg, mean ± SD; n = 8) for the respective groups, multiplied by 100.

Table 4: Yolk color values (Roche and CIELAB-values; mean ± SD; n = 8) of the egg yolks at the end of the supplementation period (day 21) for the different microalgal n-3 PUFA supplementation doses.

		Colorimeter		
Microalgal n-3 PUFA (mg/100 g feed)	**Roche value**	***L****	***a****	***b****
0	12 ± 1	65.7 ± 3.8^{cd}	7.7 ± 3.2^{a}	46.2 ± 5.6^{ab}
30	12 ± 0	66.5 ± 2.3^{d}	11.1 ± 1.3^{a}	51.1 ± 1.9^{b}
60	13 ± 1	63.0 ± 4.9^{bcd}	13.8 ± 4.4^{ab}	51.5 ± 5.4^{b}
90	13 ± 0	61.4 ± 3.7^{bcd}	13.0 ± 2.0^{a}	47.4 ± 3.0^{ab}
120	13 ± 1	59.1 ± 4.0^{abcd}	13.1 ± 2.7^{a}	45.2 ± 5.0^{ab}
150	14 ± 1	58.3 ± 3.3^{abc}	19.6 ± 5.5^{b}	45.7 ± 4.2^{ab}
200	15 ± 1	55.2 ± 5.2^{ab}	20.0 ± 5.3^{b}	44.9 ± 7.3^{ab}
300	> 15	53.0 ± 8.3^{a}	20.1 ± 4.9^{b}	41.7 ± 7.9^{a}
400	> 15	51.5 ± 4.4^{a}	18.9 ± 3.0^{b}	38.6 ± 6.2^{a}

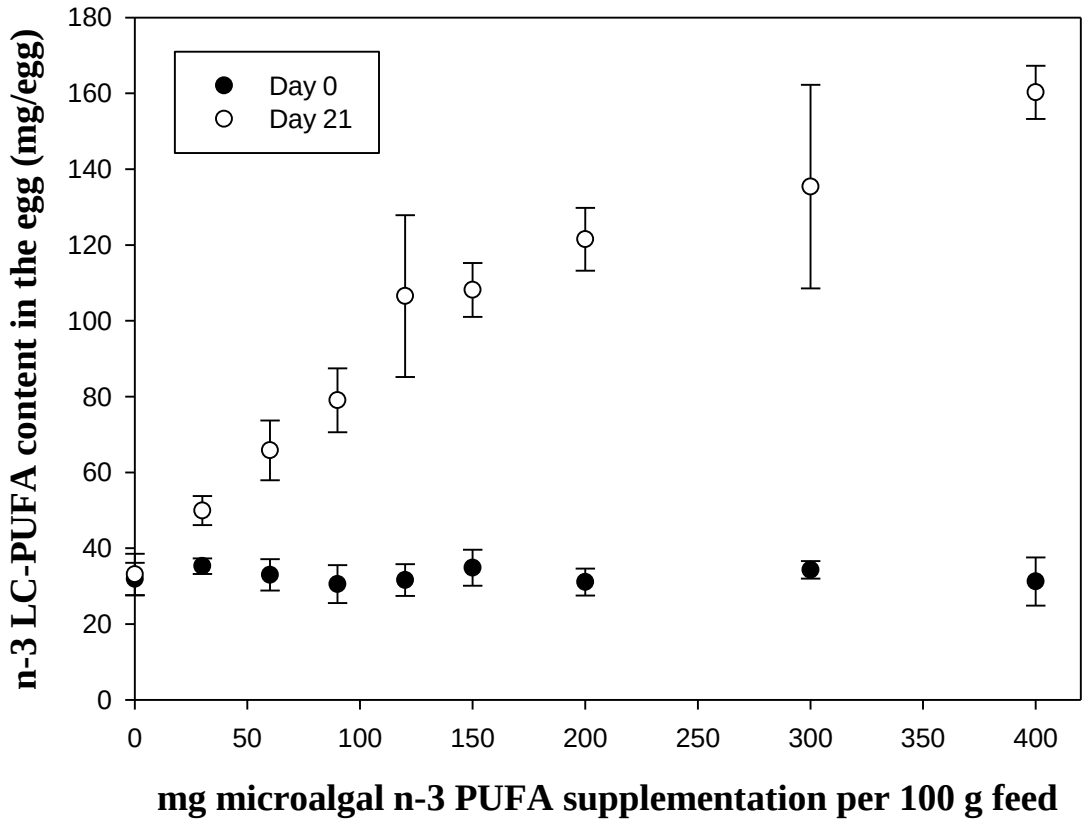
[x]Results with the same letter in the same column are not significantly different ($p < 0.05$)

Table 5: Zootechnical performance parameters and egg quality parameters during the supplementation period for the nine different supplementation doses: feed intake (in g, mean ± SD; n = 8; except for the group of 60 mg per 100 g feed, n = 7 since one hen got sick), egg production rate (in %), egg weight (in g, mean ± SD; n = 8 except, for the group of 60 mg per 100 g feed, n = 7 since one hen got sick) and yolk weight (in g, mean ± SD; n = 8, except for the group of 60 mg per 100 g feed, n = 7 since one hen got sick).

Algal PUFA (mg/100g feed)	Feed intake (g)	Egg production rate (%)	Egg weight (g)	Yolk weight (g)
0	117.2 ± 0.7^{a}	91.8	61.19 ± 0.83^{abc}	15.15 ± 1.56^{a}
30	119.1 ± 0.4^{b}	98.2	60.58 ± 0.77^{ab}	14.91 ± 0.74^{a}
60	117.3 ± 1.2^{a}	96.8	60.99 ± 1.00^{abc}	15.75 ± 0.91^{a}
90	118.9 ± 0.5^{b}	97.6	62.94 ± 1.32^{d}	15.43 ± 1.42^{a}
120	118.4 ± 0.5^{b}	98.2	61.35 ± 0.79^{abcd}	15.94 ± 0.55^{a}
150	118.8 ± 0.5^{b}	98.2	62.25 ± 0.74^{cd}	16.01 ± 0.77^{a}
200	118.8 ± 0.3^{b}	96.4	61.72 ± 1.45^{bcd}	16.10 ± 0.96^{a}
300	118.4 ± 0.8^{b}	90.5	61.59 ± 0.99^{bcd}	15.95 ± 0.85^{a}
400	116.4 ± 0.7^{a}	95.2	59.80 ± 0.78^{a}	15.70 ± 0.33^{a}

[x]Results with the same letter in the same column are not significantly different ($p < 0.05$)

375 **Figure 1:** n-3 LC-PUFA content (EPA+DPA+DHA) (in mg/egg, mean ± SD, n=8) in the egg
376 at the start and the end of the supplementation period with different n-3 PUFA doses from
377 *Isochrysis galbana*.



378